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NOVEL CELL CULTURE MEDIUM FOR USE IN OXIDATION EXPERIMENTS PROVIDES INSIGHTS INTO MECHANISMS OF ENDOTHELIAL CELL–MEDIATED OXIDATION OF LDL

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Summary

Though one prominent theory of atherogenesis involves free-radical oxidation of low-density lipoprotein (LDL) within the vessel wall by one of the vascular cell types, the mechanism for cell-mediated LDL oxidation remains unclear[sn1]. In these studies we examined the effects of media phenols, thiols, and metals on endothelial cell–mediated oxidation. We found that cell culture media such as Dulbecco modified Eagle medium and minimal essential medium are unable to support cell-mediated oxidation of LDL because they contain high concentrations of phenol red (PR) and tyrosine, both of which strongly inhibit cell-mediated oxidation. Ham's F-10, a commonly used medium for cell-mediated oxidation experiments, is also not entirely appropriate, as it contains both PR and cysteine. Cysteine is not critical for endothelial cell–mediated oxidation, but does increase oxidation of LDL in the absence of cells. Finally, of utmost importance to cell-mediated oxidation was the presence of either micromolar concentrations of Fe(II) or physiological concentrations of holo-ceruloplasmin, the protein which carries copper in plasma. An appropriate culture medium for use in cell-mediated oxidation experiments should thus contain either micromolar concentrations of Fe(II) or physiological concentrations of holo-ceruloplasmin, and should be prepared without PR, cysteine, or large concentrations of tyrosine, all of which are shown here to inhibit endothelial cell–mediated LDL oxidation. These results are consistent with a mechanism of cell-mediated oxidation involving Fenton-type chemistry and redox cycling of the metal.

Key words: atherosclerosis; lipoprotein; free radical; iron; copper.

Introduction

One of the prominent theories for the etiology of atherosclerosis is the oxidation of low-density lipoprotein (LDL) (Witzum and Steinberg, 1991; Berliner and Heinecke, 1996). According to this theory, LDL enters the vessel wall through the endothelium, where it becomes oxidized by one of the vascular cell types (Berliner and Heinecke, 1996). Part of the support for this theory is that in vitro incubation of LDL with either smooth muscle cells, macrophages, or endothelial cells in culture in media containing micromolar concentrations of free metal, such as Fe(II) or Cu(I), produces significant oxidation of LDL (Heinecke et al., 1984; Xing et al., 1998).

Free metals themselves are capable of initiating oxidation, and one theory for the development of atherosclerosis involves initiation of oxidation by metals such as Cu(I) or Fe(II) (Heinecke et al., 1984; Steinbrecher et al., 1984; Berliner and Heinecke, 1996). It is argued, however, that virtually all iron and copper in plasma is chelated to the proteins transferrin and ceruloplasmin, respectively, and these proteins are far from saturated (Aasa et al., 1963). If the argument could be made that plasma components were in a state of equilibrium, then this would indeed suggest that free metals are not present in concentrations necessary to cause LDL oxidation. What is not known is whether or not oxidizing metals exist in a free state (unchelated) in a lesion. In addition, the ability of some protein-bound metals to oxidize LDL in vitro has been demonstrated. For example, physiological concentrations of holo-ceruloplasmin (containing copper) were capable of initiating oxidation of LDL in vitro (Ehrenwald et al., 1994), as was hemin (Balla et al., 1991). Thus, possible involvement of metal-catalysis in LDL oxidation remains a plausible theory of atherogenesis.

Still other investigators have proposed the involvement of thiols such as cysteine, existing either as free amino acids or as amino acid residues on apolipoprotein B-100, in LDL oxidation (Heinecke et al., 1987; Sparrow and Olszewski, 1993). Heinecke et al. have shown that thiols such as L-cysteine promote Cu-mediated oxidation (Heinecke et al., 1993) in vitro, possibly via the enhanced production of superoxide (Winterbourn and Metodiewa, 1994, 1995).

The ultimate goal of this research is to characterize the reactive oxygen species (ROS) or other radical species that is (are) responsible for endothelial cell–mediated oxidation. In pursuit of this goal, we sought to design a minimal medium, capable of supporting cell-mediated LDL oxidation, but without any extraneous components that might interfere with the oxidation. In designing a new medium for cell-mediated oxidation of LDL we could gain information about the mechanism of cell-mediated oxidation without the use of the various inhibitors of ROS or the enzymes involved in oxidation. These inhibitors may actually inhibit oxidation by more than one

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mechanism (Jessup et al., 1991, 1993; Peterson et al., 1992), thus complicating interpretation of the results. For example, superoxide dismutase (SOD), a scavenger of superoxide, has often been used to confirm the involvement of superoxide in an oxidative process. Jessup has recently shown, however, that SOD is also an effective metal chelator (Jessup et al., 1993), thus confusing whatever conclusions could be drawn from experiments which utilize SOD to confirm the involvement of superoxide. Inhibitors of enzymes involved in oxidation, such as those that inhibit nitric oxide synthetase and lipoxygenase, have been shown to be nonspecific and to inhibit oxidation even in the absence of cells (Jessup et al., 1991; Peterson et al., 1992).

In this paper, we thus examined the role of free metals and metal chelates such as ceruloplasmin and transferrin, and free cysteine in the medium on endothelial cell–mediated oxidation of LDL and the possible interference of the oxidation by phenols in the media. The data presented here show that oxidation of LDL in the presence of endothelial cells requires metals, either free or chelated, does not require cysteine, and is inhibited in the presence of tyrosine and phenol red (PR). This work thus defines an appropriate medium for endothelial cell–mediated oxidation experiments and provides insights into the mechanism of endothelial cell–mediated oxidation of LDL.

Materials and Methods

Materials. Amino acids, ferrous sulfate heptahydrate, human transferrin containing 300–600 μg/g Fe, ferric nitrate nonahydrate, glutamine (200 m*M* solution), sodium pyruvate (100 m*M*), sodium bicarbonate (Hybrimax®, 7.5% solution), HAT (5 m*M* hypoxanthine, 0.02 m*M* aminopterin, 0.8 m*M* thymidine) media supplement (HybriMax®, 50×) for hybridoma cells, gentamycin (10 mg/ml), and trypsin·ethylenediamine tetraacetic acid (EDTA) were all of tissue culture grade and were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine ceruloplasmin (solution in 0.2 *M* NaCl and 0.05 *M* sodium acetate, containing 80–120 μg/ml Cu), horse heart cytochrome *c,* human erythrocyte SOD, and *N*-ethylmaleimide were also obtained from Sigma. PR (0.5% solution) was obtained from GIBCO (Grand Island, NY). Dulbecco's phosphate-buffered saline (DPBS, cellgro®) and heat-inactivated fetal bovine serum (FBS, cellgro®) were obtained from Mediatech (Herndon, VA). Ham's F-10 and Dulbecco modified Eagle medium (DMEM) were obtained from BioWhittaker (Walkersville, MD). Powdered DMEM (cat. no. D-2902) and Hank's buffered salts (cat. no. H-1387), both containing no PR or sodium bicarbonate, and powdered minimal essential medium (MEM, cat. no. M-3024, containing no PR, sodium bicarbonate, or glutamine) were all obtained from Sigma. Reagents used for protein or oxidation assays, including Folin and Ciocalteu's phenol reagent, lauryl sulfate (sodium dodecyl sulfate), xylenol orange (3,3′-bis[*N*,*N*-di(carboxymethyl)-aminomethyl]-*o*-cresol-sulfonephthalein), and butylated hydroxytoluene (2,6-di-*t*-butyl-*p*-cresol) were also purchased from Sigma.

Methods. For isolation of LDL, plasma was collected in EDTA from fasted normolipidemic donors and was either stored at −20° C or was used immediately. At the start of each experiment, lipoproteins were isolated from plasma by the sequential density gradient ultracentrifugation method described by Hatch and Lees (1968). LDL was identified as the fraction with a density of 1.019–1.063 g/ml. Once the LDL was isolated, it was used immediately. Before incubation with cells, the LDL was desalted extensively, first using two Pharmacia Biotech Sephadex® G-25 M (PD-10) gel filtration columns in sequence, then by exhaustive dialysis against 0.15 *M* saline at 4° C and in the dark. Finally, protein was determined by the modified Lowry protein assay (Markwell et al., 1978), and the LDL was diluted to 0.25 mg/ml in the appropriate culture medium.

For preparation of specialized cell culture oxidation media for incubation with LDL the following protocols were used. (1) DMEM+ (formulated to contain the components of DMEM plus cysteine and metals as contained in Ham's F-10). Powdered DMEM (containing no PR) was diluted in 1000 ml deionized distilled water. Stock solutions (100×) of the additional medium components were then prepared and added to the DMEM such that the final concentrations were as follows: 0.00834 mg/ml ferrous sulfate·7 H_2O, 0.000025 mg/ml cupric sulfate·5 H_20, and 0.000288 mg/ml zinc sulfate·7 H_2O. Solid sodium bicarbonate, 1.2 g, 0.127 g L-arginine·HCl, 0.35 g L-cysteine·HCl, and 4.1 mg hypoxanthine were then added, and the solution thus prepared was filtered through a 0.22-μm filter. (2) MEM+ (formulated to contain the components of MEM plus cysteine and metals and lacking tyrosine and PR). Powdered MEM (without amino acids) was diluted with 1000 ml deionized distilled water. The solution was then sterile-filtered as described above and 4.6 ml 200 m*M* glutamine and 6.95 ml 100 m*M* sodium pyruvate, both of which were purchased as sterile solutions, were added. This solution was then diluted 1:25 v/v with DPBS. Stock solutions (100×) of Fe(II), Cu(II), and Zn were then added as described before. An aliquot of a 100× stock solution of essential amino acids was also added. This essential amino acid solution was composed of 21.1 mg/ml L-arginine·HCl, 1.93 mg/ml L-histidine·HCl, 1.102 mg/ml L-leucine, 2.64 mg/ml L-lysine·HCl, 4.42 mg/ml L-methionine, 0.368 mg/ml L-phenylalanine, 0.165 mg/ml L-threonine, 0.02 mg/ml L-tryptophan, 0.166 mg/ml L-valine, 0.408 mg/ml hypoxanthine, and 35 mg/ml L-cysteine·HCl. The resulting medium was again sterile-filtered. *Note* that commercially available amino acid solutions could not be used, because they contain large concentrations of tyrosine (a nonessential amino acid). (3) MEM2 (formulated to contain the components of MEM without tyrosine and PR but with metals). Powdered MEM (without amino acids) was diluted in 1000 ml deionized distilled water, was sterile-filtered, and was diluted 1:25 v/v in sterile DPBS. Stock solutions (100×) of Fe(II), Cu(I), Zn, and the essential amino acids (as described above, except this stock solution contained no cysteine) were added as described previously. Finally, 4.6 ml 200 m*M* L-glutamine and 6.95 ml 100 m*M* sodium pyruvate were added. This solution was thus basically the same as MEM+, except that MEM2 contained no cysteine. For media in which metals were deleted, no Cu(II) or Fe(II) was added to MEM2. For media in which all thiols were excluded, the 100× amino acid stock solution was prepared without L-methionine. *Note*, however, that a small amount of methionine (0.0006 mg/ml) would still be present in this medium, since the original MEM solution before the 1:25 dilution contained methionine.

TABLE 1

COMPARISON OF PERTINENT COMPONENTS OF THE VARIOUS MEDIA USED

	PR	Tyr	Cys	Fe	Cu	Zn
Ham's F-10	+	l0	+	+	+	+
DMEM	+	+	−	−	−	−
DMEM+	+	+	+	+	+	+
MEM	+	+	−	−	−	−
MEM+	−	−	+	+	+	+
MEM2	−	−	−	+	+	+

The essential components of both the commercially available and specially prepared media are summarized in Table 1.

The EaHy-1 cells (human aortic endothelial cells immortalized by hybridization to cells from a human adenoma) used in these experiments were previously shown to maintain their characteristic cobblestone morphology, presence of factor VIII–related antigen, and ability to take up acetylated LDL (Navab et al., 1988, 1991; Reaven et al., 1994). These endothelial cells were grown and maintained in DMEM medium containing 10% FBS, 0.125 mg/ml gentamycin, 4.5 mg/ml glutamine and HAT medium supplement (50×, dissolved in 10 ml deionized water and diluted 1:50 in the DMEM). Before the start of the oxidation experiment, cells were plated into six-well plates and were allowed to grow for 2–3 d, or until confluent. DMEM was removed and the cells were washed with sterile PBS. The cells were next incubated at 37° C with 2 ml per well of 0.25 mg/ml LDL in a serum-free, oxidation medium (prepared as described) for 0–36 h. As a control for the spontaneous oxidation of LDL that might result from incubation at 37° C in the absence of cells, the 0.25 mg/ml LDL was also incubated in wells without cells at 37° C for 0–36 h. Following incubation with the cells, aliquots of medium were collected and were stored at −20° C for less than 1 wk.

Lipid oxidation was assessed using the FOX (ferrous oxidation/xylenol or-

TABLE 2

ENDOTHELIAL CELL–MEDIATED OXIDATION OF LDL IN VARIOUS MEDIA[a]

Medium	nmol FOXRS/mg protein	
	Without cells	With cells
Ham's F–10	137 ± 41	382 ± 51
DMEM	0	0
DMEM+	0	0
MEM	0	0
MEM+	0	0
MEM2	145 ± 41	405 ± 49

[a] Data represent the mean ± SEM.

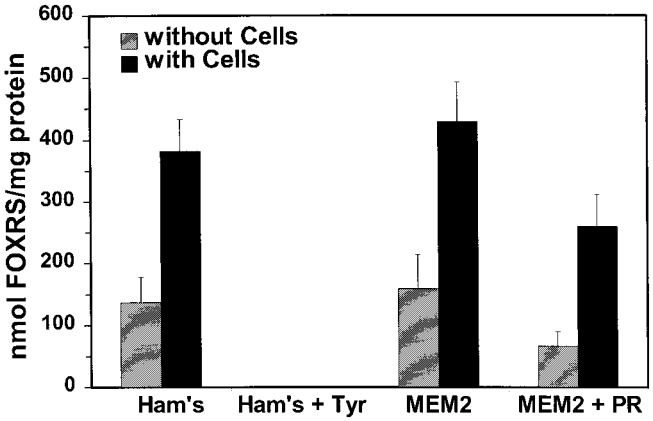

FIG. 1. Determination of the media components of Ham's F-10 and DMEM which inhibit oxidation. Lipid hydroperoxides, formed in each of the LDL-containing media incubated with endothelial cells, were assessed by measuring nmol FOXRS/mg protein using the FOX assay. *Note* that the concentration of tyrosine added to Ham's F-10 was equivalent to that present in DMEM. Likewise, the concentration of PR added to MEM2 was equal to that present in Ham's F-10. Data represent the mean ± SE.

ange) (Jiang et al., 1992) assay, which measures the endpoint of lipid oxidation, lipid hydroperoxides. Both the samples of LDL incubated in the presence of endothelial cells and the LDL incubated without cells were assayed for lipid oxidation.

Before performing the FOX assay, the lipid hydroperoxides were extracted from 100 μL of the LDL-containing medium by adding 0.9 ml of methanol as described by Jiang et al. (1992). After centrifugation at 12,000 × *g* for 3 min to remove precipitated protein, the extracts were evaporated under nitrogen and were redissolved in 100 μl 9:1 methanol:water (v/v) solution. To perform the FOX assay, 0.9 ml of the FOX reagent (Jiang et al., 1992) was added to 0.1 ml of either the sample or dilutions of 30% hydrogen peroxide used as a standard. Samples and standards were allowed to develop for 30 min, after which absorbance was measured at 560 nm.

Superoxide concentrations present in various media containing no LDL and incubated at 37° C with and without endothelial cells were measured using the cytochrome *c* assay (Azzi et al., 1975). Aliquots of media were collected before and after 24 h incubation periods. To perform the assay, 0.3 ml of the media samples were immediately aliquoted and were added to either 0.1 ml deionized water or to 0.1 ml 0.5 mg/ml SOD. After 5 min, 0.1 ml 400 μ*M* cytochrome *c* was added to all samples and absorbance was measured at 550 nm. In some experiments, wells with and without cells and ~2 ml media were incubated with either 0.2 ml SOD or 0.2 ml sterile deionized water for 24 h. Superoxide concentration was then determined by taking aliquots of 0.4 ml media before and after the incubation period, adding 0.1 ml 400 μ*M* cytochrome *c*, and measuring the absorbance at 550 nm. Superoxide concentration was defined as the portion of the increase in A_{550} that was inhibitable by excess SOD, and was calculated using a molar absorptivity of 21.1/m*M*/cm. Since superoxide is an unstable radical, these measurements reflect the concentration of superoxide present at the time of measurement and not the total amount of superoxide produced over the 24-h period.

RESULTS

As the data in Table 2 clearly show, LDL diluted in PR-free DMEM incubated with endothelial cells remained unoxidized at 36 h. LDL was oxidized dramatically, however, when incubated with cells and in Ham's F-10 medium, a medium commonly used for cell-mediated oxidation experiments. Though there was also significant oxidation of LDL incubated in Ham's F-10 in the absence of cells, at least twofold more oxidation resulted when LDL was incubated with cells. When DMEM was further modified so as to contain some of the components normally present in Ham's F-10 but not in DMEM, such as Fe(II), Cu(I), L-cysteine, Zn, and additional arginine, still no oxidation was observed in LDL incubated either with or without cells. MEM and MEM+ (modified in the same way as DMEM+) were also incapable of oxidizing LDL either cell-free or in the presence of cells. Since DMEM contains 50-fold more tyrosine, a known scavenger of ROS, than Ham's F-10 and MEM, 25-fold more tyrosine, a new medium was prepared (designated MEM2) in which MEM was first diluted 1:25 v/v with DPBS. Glutamine (0.146 g/L), sodium pyruvate (0.11 g/L), the essential amino acids (with no added cysteine or tyrosine), hypoxanthine, Fe(II), Cu(II), and Zn were added at the concentrations normally present in Ham's F-10. Oxidation comparable to that observed in Ham's F-10 medium was observed for LDL incubated in MEM2 both cell-free and in the presence of cells.

Since the only other prominent differences between these two media were the presence of PR and tyrosine, in another experiment, a possible involvement of these two media components in preventing cell-mediated oxidation of LDL was investigated. In this experiment, comparable oxidation was observed (both with and without cells) in the presence of either MEM2 or Ham's F-10 (Fig. 1). Addition of PR to MEM2 (such that the concentration of PR was equal to that in Ham's F-10) inhibited both cell-free and cell-mediated oxidation. Similarly, addition of tyrosine to Ham's F-10, such that the concentration of tyrosine was equal to that in DMEM, completely eliminated oxidation both in the presence and absence of cells.

Figure 2 shows the results of experiments in which Fe(II) and Cu(II), either together or individually, were excluded from MEM2. In the absence of both Fe(II) and Cu(II) virtually no LDL oxidation was observed, both in the presence and absence of cells. Addition of Cu(II) in the nanomolar amounts normally present in Ham's F-10 did not significantly catalyze LDL oxidation, but addition of Fe(II) (3 μ*M*) initiated both cell-mediated and cell-free oxidation. Furthermore, this oxidation was comparable to that observed for LDL incubated in MEM2 (containing both Fe[II] and Cu[II]). *Note* that the concentration of Fe and Cu used in these experiments were the same as those present in Ham's F-10, even though the concentration of ferrous sulfate is much larger than that of copper sulfate. These concentrations were actually experimentally instrumental, as Cu(II) is a much stronger oxidant of LDL than Fe(III) (Tribble et al., 1996).

In similar experiments, LDL was incubated with and without cells in MEM2 prepared in Hank's buffered salts, in which physiological concentrations of either ceruloplasmin (containing 18 μ*M* copper) or transferrin (containing 21.5 μ*M* iron) were added in place of both Fe(II) and Cu(II) (Fig. 3). Furthermore, each medium containing

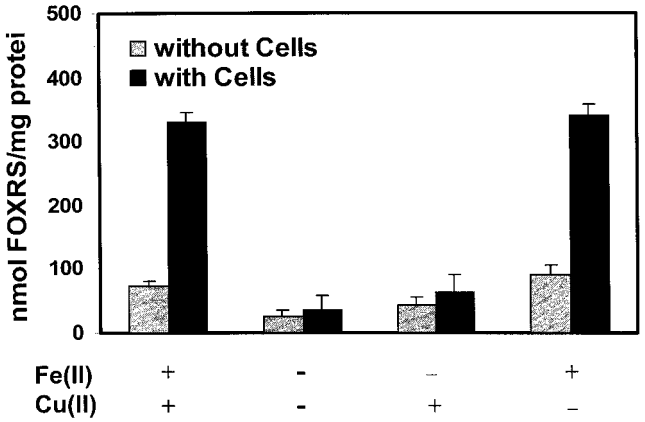

FIG. 2. Determination of the role of metals in endothelial cell–mediated LDL oxidation. The media components Fe(II) and Cu(II) were investigated for their potential to catalyze cell-mediated LDL oxidation. The concentrations of Fe(II) and Cu(II) used in these experiments were the same as those present in Ham's F-10. Lipid hydroperoxides, formed in each of the LDL-containing media incubated with cells, were measured as nmol FOXRS/mg protein using the FOX assay. Data represent the mean ± SE.

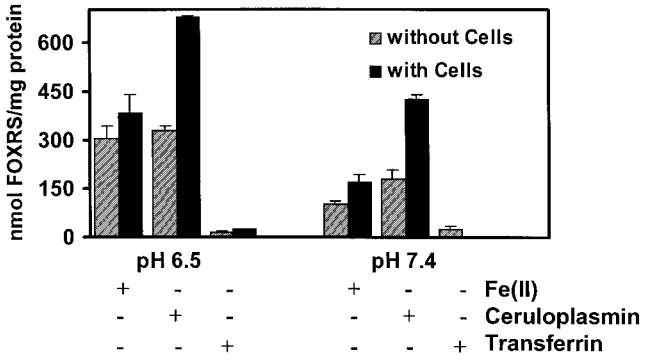

FIG. 3. Determination of the ability of metal chelates to initiate endothelial cell–mediated LDL oxidation and the influence of pH on this initiation. Fe(II), ceruloplasmin, and transferrin were added to the medium at either the concentration present in Ham's F-10 (for Fe[II]) or at physiological concentrations (for ceruloplasmin and transferrin). Each were incubated in media at either pH 6.5 or 7.4. Lipid hydroperoxides, formed in each of the LDL-containing media incubated with endothelial cells, were assessed by measuring nmol FOXRS/mg protein using the FOX assay. Data represent the mean ± SE.

either Fe(II), ceruloplasmin, or transferrin was divided into two subsets, one maintained at pH 6.5 (by addition of sodium bicarbonate), and the other, pH 7.4. Ceruloplasmin was capable of catalyzing both cell-free and cell-mediated oxidation at both pH 6.5 and 7.4, but more oxidation was observed at pH 6.5. In addition, in one preliminary experiment, when LDL was incubated with MEM2 in the presence of 30-fold less ceruloplasmin, oxidation was observed at pH 6.5, but not at pH 7.4 (data not shown). Transferrin, at either pH, was unable to catalyze either cell-free or cell-mediated oxidation.

The influence of amino acid thiols, such as cysteine and methionine, in the medium was also investigated. MEM2 was prepared with and without either methionine, cysteine, or both, and was incubated with and without endothelial cells for 36 h. In these experiments, methionine had no impact on LDL oxidation either with or without cells (Fig. 4). Cysteine increased oxidation observed in

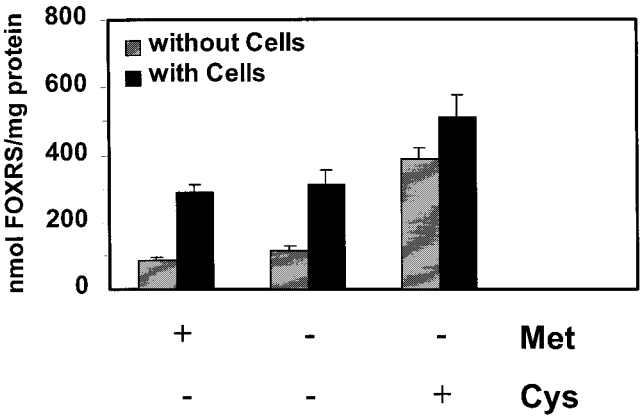

FIG. 4. Role of cysteine in endothelial cell–mediated oxidation. To investigate the role of the thiol media components methionine and cysteine, each were added (0.044 and 0.35 mg/ml, respectively) to MEM2 media and lipid hydroperoxides were measured as nmol FOXRS/mg protein using the FOX assay. Data represent the mean ± SE.

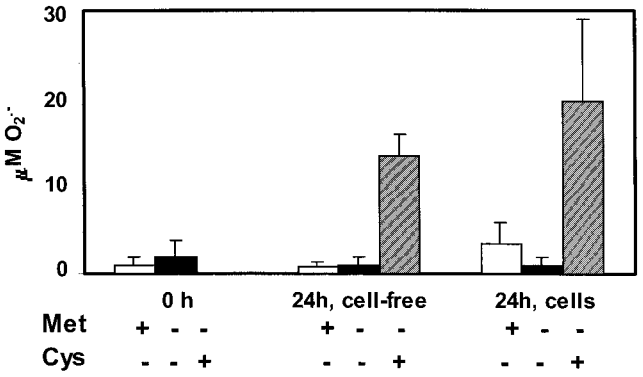

FIG. 5. Superoxide concentration in various media with or without cysteine and methionine. *Note* that the cells were incubated in each of the media (containing no LDL) prepared with and without cysteine or methionine and superoxide was assessed by measuring cytochrome *c* reduction. Data represent the mean ± SD.

the presence and absence of cells. Since cysteine is known to interact with metals to form superoxide, a possible role of superoxide in the increased non–cell-mediated oxidation was also investigated. As such, superoxide was measured in each of these media (containing no LDL) and in the presence and absence of cells (Fig. 5). The presence of cysteine in the media led to marked production of superoxide in both the absence and presence of cells. *Note* that in the presence of LDL, we were unable to measure superoxide using the cytochrome *c* assay (data not shown).

DISCUSSION

Incubation of LDL in Ham's F-10 with a human aortic endothelial cell line (EaHy-1) produced significant oxidation of LDL (Table 2). Furthermore, as we have previously shown, this oxidation can be inhibited by enrichment of the LDL with antioxidants such as α-tocopherol and β-carotene (Dugas et al., 1998). Still uncertain, however, is the mechanism of endothelial cell–mediated oxidation. Studies by other investigators, aimed at elucidating this mechanism, focussed on the use of inhibitors of either the formation of ROS or

the enzymes involved in lipid oxidation (Hiramatsu et al., 1987; Parthasarathy et al., 1989; Jessup et al., 1992). As presented in the "Introduction," nearly all of these inhibitors have since been proven nonspecific and inhibit oxidation by more than one mechanism (Jessup et al., 1991, 1993; Peterson et al., 1992), and thus the use of such inhibitors for the purpose of determining the mechanism of cell-mediated oxidation is problematic. In order to circumvent the use of these inhibitors, we began by creating various media de novo such that we might determine which components are essential to the oxidation, and conversely, which components interfered with the oxidation.

PR and tyrosine interfere with endothelial cell–mediated oxidation. Ham's F-10, but not DMEM or MEM, supports endothelial cell–mediated oxidation, even when components unique to Ham's F-10 were added to DMEM or MEM (DMEM+ and MEM+, respectively). However, when the concentration of tyrosine in MEM was reduced 25-fold (MEM2) such that the amount of tyrosine in MEM was equal to that in Ham's F-10, a similar amount of both cell-free and cell-mediated oxidation was observed in this medium as compared to oxidation in Ham's F-10. Tyrosine is thus a potent inhibitor of cell-mediated oxidation. This is not unexpected, since tyrosine is a known scavenger of reactive oxygen species (Maskos et al., 1992). The high concentration of PR in DMEM and MEM is likely another contributing factor to the inability of DMEM and MEM to support cell-mediated oxidation, as addition of PR to Ham's F-10 or MEM2 dramatically inhibited oxidation (Fig. 1).

It is not surprising that PR serves as an antioxidant in these media, as PR is a biphenolic compound, and compounds of this class (i.e., vitamin E, butylated hydroxytoluene, butylated hydroxyanisole, etc.) are known scavengers of lipid peroxyl radical (Halliwell and Gutteridge, 1985). Since oxidation experiments performed in media containing no PR or other components that interfere with oxidation would be desirable, specialty media prepared containing no PR should be considered. It is also problematic that all specialty media containing the essential amino acids also contain tyrosine, a nonessential amino acid. Furthermore, in most of these media, the concentration of tyrosine is large (e.g., DMEM and MEM). Even the commercially available solutions of essential amino acids used for the purpose of preparing media contain a similarly large concentration of tyrosine.

Involvement of free cysteine. Though it has been shown that cysteine is critical to smooth muscle cell–mediated oxidation (Heinecke et al., 1987, 1993), we find no such requirement for endothelial cell–mediated oxidation (Fig. 3). Cysteine does, however, increase LDL oxidation in the absence of cells (Fig. 3). This is likely due to its reaction with metals in the medium to produce superoxide (Winterbourn and Metodiewa, 1994). As stated earlier, the resulting superoxide might then be dismutated by SOD to produce H_2O_2, which, in turn, could initiate Fenton-type metal-mediated oxidation of LDL.

Requirement for free or chelated metals and proposed mechanism of endothelial cell–mediated oxidation of LDL. Of crucial importance to endothelial cell–mediated oxidation was the presence of metals (Fig. 2). We experimented with the addition of either copper sulfate or ferrous sulfate to the medium denoted here as MEM2. It is well-known that the addition of Fe(II) to aqueous solution results in its rapid oxidation to Fe(III). Nevertheless, since these two media components are what is normally used in the formulation of Ham's F-10, each were added as is, so that we might assess the role of these media components in the oxidation. Furthermore, they were added at the concentrations normally present in Ham's F-10, even though the concentration of ferrous sulfate is much larger than that of copper sulfate.

It is possible that the mechanism for endothelial cell–mediated oxidation in cell culture medium is as follows. First of all, these endothelial cells produce superoxide, even when no LDL is present in the medium (Fig. 5). When the Fe(II) is dissolved in the aqueous medium, it is rapidly oxidized to Fe(III). However, the Fe(III) thus produced, together with the superoxide produced by the cells can react (as shown in equation 1) to form Fe(II) and oxygen. The Fe(II) can then react with H_2O_2 (Winterbourn, 1995), either produced by the cells or from the

$$\mathrm{Fe(III)} + \mathrm{O_2}\cdot^- \longrightarrow \mathrm{Fe(II)} + \mathrm{O_2} \quad (1)$$

$$\mathrm{O_2}\cdot^- + 2\mathrm{H}^+ \xrightarrow{\text{SOD}} \mathrm{H_2O_2} + \mathrm{O_2} \quad (2)$$

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \longrightarrow \mathrm{Fe(III)} + \cdot\mathrm{OH} + \mathrm{OH}^- \quad (3a)$$

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \longrightarrow \mathrm{Fe(III)OH} + \mathrm{OH}^- \quad (3b)$$

dismutation of superoxide by SOD (equation 2) to produce hydroxyl radical (equation 3a), or perhaps more correctly, ferryl ion (equation 3b) (Rush et al., 1990). The chemistry described by the reactions in equations 1–3 is well known and is referred to as the Fenton Reaction (Winterbourn, 1995). (*Note* that Cu[I] is capable of the same type of chemistry; however, at the concentrations present in Ham's F-10, we do not see significant oxidation from this component.) Both hydroxyl radical and ferryl ion are very potent ROS. While the hydroxyl radical is highly reactive and lacks site specificity, the ferryl ion is much more selective and only reacts with readily oxidizable functional groups (Rush and Koppenol, 1986). What is necessary for initiation such as that described by equations 1–3 is a continuous redox cycling of the metal. Perhaps the production of superoxide by the cells is precisely what is required for the redox cycle of the iron present in the medium, which in turn leads to the production of either ferryl ion or hydroxyl radical.

It is interesting to note that while a source of metal was critical to the oxidation, these metals could exist either unchelated (such as Fe[II]) or bound to protein, such as ceruloplasmin (Fig. 2). *Note* that the concentration of ceruloplasmin used in these experiments was of physiological concentration and that the ceruloplasmin was only 30% saturated with copper. It is interesting to note that even 30-fold less than physiological amounts of ceruloplasmin produced a measurable amount of cell-mediated oxidation (data not shown), with very minimal oxidation in the absence of cells. This concentration of ceruloplasmin might actually be preferable for use in oxidation experiments, since the higher concentrations also resulted in significant cell-free oxidation.

Several researchers have argued that although metals can be used to oxidize LDL in vitro by either cell-mediated or non–cell-mediated processes, metal-mediated oxidation would not be expected to occur in vivo (Heinecke, 1997). Their reasoning is that since, at equilibrium, all metals in the blood are found chelated to protein, and since the binding sites of these proteins are far from saturated, no free metal would be available for oxidation (Aasa et al., 1993). Though this might be true of metals and chelates at equilibrium, this does not rule out the possibility that the metals might at some point be made available for initiating oxidation. In fact, Smith et

al. have demonstrated a substantial amount of "catalytic activity" of metals in atherosclerotic lesions (Smith et al., 1992). In their experiments, free (not bound to protein) Fe and Cu were measured using the bleomycin and phenanthroline assays, respectively. These metals were also shown to enhance the production of hydroxyl radical from hydrogen peroxide and in the presence of ascorbate, further strengthening the argument that metals in atherosclerotic lesions are available for redox reactions. It is also possible that metal chelates themselves may participate in cell-mediated LDL oxidation. Ehrenwald and Fox, for example, have shown that copper bound to ceruloplasmin was capable of producing significant LDL oxidation when the LDL was incubated with ceruloplasmin in vivo (Ehrenwald et al., 1994).

We in fact report here that copper-chelated ceruloplasmin is capable of catalyzing a substantial amount of endothelial cell–mediated oxidation of LDL. Xing et al. have also recently shown that a metal chelate known as Zop (opsonized zymozan) is capable of initiating macrophage-mediated LDL oxidation (Xing et al., 1998). These data taken together thus indicate that metal-assisted cell-mediated LDL oxidation within the artery wall is indeed possible, even if the metals are chelated.

Leake has argued that the reduced pH of atherosclerotic lesions not only enhances the rate of the various reactions that occur during lipid oxidation, but should also release chelated metals to make them available for initiating oxidation (Leake, 1997). Reduced pH, however, was not necessary for cell-mediated oxidation in the presence of ceruloplasmin. Conversely, it was incapable of releasing the iron in transferrin for initiation of cell-mediated oxidation. It is likely that the iron in transferrin is bound more tightly than the copper in ceruloplasmin. We do not argue, however, that ceruloplasmin is thus the principle oxidant in the vessel wall, as environmental conditions within the vessel wall other than pH might result in the release of iron in transferrin.

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